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OPTIMAL RESOLUTION OF EYE LENS γ-CRYSTALLINS BY CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON SYNCHROPAK CM300

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SUMMARY

Cation-exchange high-performance liquid chromatography on SynChropak CM300 in Tris–acetate buffers of pH 5–7, using sodium acetate gradients, produces an excellent separation of the various γ-crystallin gene products and their post-synthetically modified forms from eye lens. With a single analysis of total lens extract, the γ-crystallins can be resolved, quantified and collected for amino acid analysis.

Experimental conditions are presented for optimal resolution of individual human, rat, bovine and dogfish shark γ-crystallins. Applications presented include determinations of differential synthesis of γ-crystallins and chemical modification (oxidation by hydrogen peroxide) *in situ*.

INTRODUCTION

γ-Crystallins are structural proteins of vertebrate eye lenses which play a critical role in lens transparency and cataract development[1–3]. Up to six different γ-crystallin gene products, of greater than 70% sequence homology[4–6], are differentially synthesized during lens growth *in vivo*[3,7,8]. Upon cataract formation or aging of γ-crystallins *in vivo*, post-translational modification generates additional species which are generally more acidic and which contain disulfides[1,2,9,10].

Separation and purification of individual gene products is difficult, since nearly all γ-crystallins have similar molecular weights of about 21 000 and isoelectric points near pH 8.0[9–12]. Partial resolution has been obtained by low-pressure cation-exchange chromatography on sulphopropyl (SP)-Sephadex at pH 5–6[3,7,9,11,12], and the order of elution of γ-crystallins is apparently related to the number of surface-exposed histidine residues[3,13]. A more accurate and rapid procedure for the identification and quantification of the many gene products and their modified forms should provide valuable insight into the processes of differential synthesis, aging and cataract formation in the eye lens.

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In a preliminary communication we reported that cation-exchange high-performance liquid chromatography (HPLC) on SynChropak CM300 provides a superior resolution of bovine γ-crystallins[14]. In the present paper we present optimal conditions for separation of the multiple γ-crystallins from bovine, rat, dogfish and human lens. Identification of components is based on comparison of amino acid compositions with known sequences of the gene products.

EXPERIMENTAL

Lens extraction

Eye lenses were obtained as described before from 2–6 months old humans[15], 20–30 years old dogfish[16], 15 days old rat[3] and 7 days to 5 years old cow[7,14,17]. Whole lenses were used, except for the five-year old bovine lens which was carefully dissected into nine concentric layers. Whole lenses or lens microsections were extracted in either HPLC mobile phase buffer A or 0.05 *M* sodium phosphate buffer, pH 7.0 (containing 1 m*M* EDTA, 0.1 m*M* dithiothreitol and 0.02% sodium azide). The extracts were centrifuged at 10000 *g* for 20 min at room temperature. Supernatants were stored at 4°C and dialyzed against appropriate buffers just prior to subsequent chromatographic analysis, if so required.

Low-pressure chromatography

Monomeric crystallins (*i.e.* $\gamma + \beta_s$) were isolated by Sephadex G-75 chromatography of the extract in the same phosphate buffer, pH 7.0, at room temperature[7,10]. Low-pressure cation-exchange chromatography of monomeric crystallins was performed on a SP-Sephadex C-50 column (150 × 10 mm) in 0.275 *M* sodium acetate buffer, pH 4.8, with a linear 0 to 0.2 *M* sodium chloride gradient.

Cation-exchange HPLC

Ion-exchange HPLC was carried out at room temperature in a prepacked SynChropak CM300 column, 250 × 4.1 mm, connected to a SynChropak CSC precolumn, 50 × 4.1 mm (SynChrom, Lafayette, IN, U.S.A.). This CM300 material consists of 6.5 μm silica beads with a polyamide coating, derivatized with carboxymethyl groups, and a 300 Å average pore size.

The HPLC system consisted of a Beckman 421 controller, two Beckman 110A pumps, an Altex 210 injector with a 20-μl loop, and either a Beckman 160 detector (280 nm) with Hewlett-Packard 3390A integrator or a Hewlett-Packard 1040A diode-array detector with a 7999A workstation. Elution was at 1 ml/min, 1000–1200 p.s.i., using mobile phase buffers 0.02 *M* Tris–acetate (A) and 0.02 *M* Tris–acetate, 0.5 *M* sodium acetate (B), both containing 1 m*M* EDTA, 0.1 m*M* dithiothreitol and 0.02% sodium azide, and both adjusted to the appropriate pH (range 5–7). Salt gradients are given in the figure legends; each run was followed by 5 min in 100% buffer B and reequilibration for at least 15 min in the starting mixture of A and B. HPLC-grade water (Milli-Q) was used throughout.

γ-Crystallin solutions (or lens microsection extracts) were dialyzed against HPLC buffer A, reconcentrated by ultrafiltration (Amicon, YM-10 membrane), and centrifuged prior to HPLC. Typically the 20-μl sample injected contained about 40 μg γ-crystallins or about 500 μg total lens proteins (containing about 100 μg γ-crys-

tallins). Individual γ-crystallin subfractions in the HPLC eluate were collected from several runs and subjected to amino acid analysis.

Amino acid analysis

Protein samples were hydrolyzed in 6 *M* hydrochloric acid at 110°C for 24 h in evacuated tubes and subsequently analyzed in duplicate on a Dionex D-500 amino acid analyzer. Threonine and serine value were corrected upward by 10% and 20%, respectively, to account for losses during hydrolysis. Cysteine and tryptophan were not determined.

Lens incubation with hydrogen peroxide

Fresh calf lenses (age 1–2 weeks), lens weight 0.9–1.1 g, were incubated in 5 ml 0.1 *M* sodium phosphate, 0.02% sodium azide, pH 7.0, to which appropriate amounts of 30% hydrogen peroxide (Mallinckrodt, analytical-reagent grade) were added at time zero, for 20–24 h at room temperature (21°C) unless specified otherwise[18].

Lenses were subsequently washed for 3–24 h with several changes of phosphate buffer to remove residual hydrogen peroxide, prior to extraction.

RESULTS AND DISCUSSION

Optimal resolution conditions

Rat. All six rat γ-crystallin genes are known, and the corresponding amino acid sequences have been deduced[6]. Fig. 1 illustrates the SynChropak CM300 elution pattern at pH 6.0 of total lens extract from 15-days old rats. The multimeric α- and β-crystallins are more acidic than the γ-crystallins and do not bind to the cation-exchanger under these conditions. With a salt gradient, six peaks are eluted which have been identified by amino acid analysis[3] as the six γ-crystallin gene products, as shown in Fig. 1 (nomenclature in ref. 6).

The resolution of these six peaks is excellent, and no cross-contamination oc-

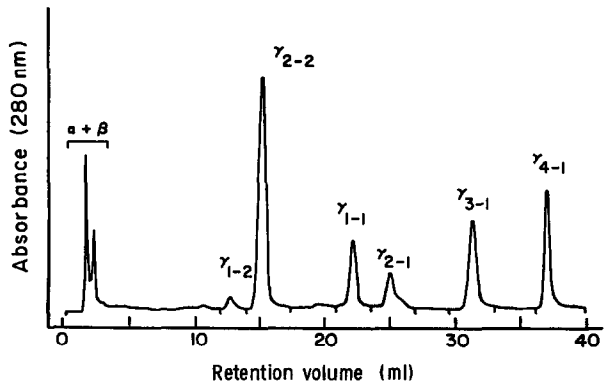

Fig. 1. Cation-exchange HPLC on SynChropak CM300 of rat γ-crystallins. Total lens extract from rats (15-days old) was applied. Elution conditions: Tris–acetate, pH 6.0; 5% mobile phase B isocratic for 2 min, 5 to 15% B linear gradient in 10 min, 15% B isocratic for 10 min, and 15 to 40% B linear gradient in 15 min; flow-rate, 1 ml/min.

curs. The order of elution is not so much determined by isoelectric points, since these are nearly identical for all six rat γ-crystallins[12], but rather by the number of exposed His residues which is 4, 5, 4, 6, 8 and 9, respectively, for the components shown in Fig. 1[3,13], and by their p*K* values. The peak elution order on SynChropak CM300 (at pH 6.0) differs from that on SP-Sephadex (at pH 4.8) in that the $\gamma_{1\text{-}1}$ and $\gamma_{2\text{-}2}$ peaks are switched[3]. This dependence of elution order on pH was also found for bovine γ-crystallins[14], and it must reflect differences in p*K* values of individual His residues in the pH 5–7 range. This phenomenon becomes an important tool when attempting to separate γ-crystallins with the same number of exposed His residues. In such a case, varying both the pH and salt gradient generally allows one to find conditions for good resolution.

Remarkable is the excellent separation in Fig. 1 of rat $\gamma_{3\text{-}1}$ and $\gamma_{4\text{-}1}$ which differ in only 4 of 173 residues[6]. This is understandable, however, when we realize that two of the substitutions involve charged residues (^{79}Arg to His, ^{148}Gly to Arg) resulting in an additional exposed His residue.

Using this CM300 cation-exchange HPLC procedure, the spatial and temporal distribution of γ-crystallins in 1-day to 420-day rat lens has been determined[3]. These data have provided essential insight into the processes of differential synthesis and aging of rat γ-crystallins *in vivo*. For instance, we demonstrated that all six gene products are indeed synthesized, but in varying ratios during development, and that little charge modification of these γ-crystallins occurs in normal rat lens in the first 420 days of life.

Dogfish. Dogfish represents an example in which none of the γ-crystallin genes are known. Hence, pure protein chemistry must be employed to estimate the number of primary gene products synthesized and their post-translational modified forms. Dogfish total γ-crystallins were first separated from α- and β-crystallins on Sephadex G75[16]. Subsequently, using the traditional low-pressure cation-exchange chromatography on SP-Sephadex, we partially resolved five γ-crystallin subfractions (I–V, Fig. 2a). Isoelectric focusing indicates, however, that none of these subfractions represents a single component[16].

Indeed, using the high resolving power of SynChropak CM300 at pH 6.0 (Fig. 2b), it becomes clear that fractions I, III, IV and V all consist of multiple γ-crystallins. Although SynChropak CM300 provides an excellent separation of more than ten components under these conditions, several peaks are still asymmetric or incompletely resolved. This implies that additional variations in pH and salt gradient are required for further purification of all the individual γ-crystallin components. Amino acid compositions of all the subfractions were very similar, yet distinct in the sense that at least three subclasses could be distinguished, possibly derived from three different gene products[16]. Many of the CM300 peaks could represent age-modified γ-crystallins since 20–30 years old dogfish were used. Clearly, a very complex mixture of γ-crystallins is present in these old dogfish lenses. Nevertheless, SynChropak CM300 chromatography provides an excellent means for the purification and quantification of individual γ-crystallins.

Human. Six human γ-crystallin genes are known, two of which are pseudogenes that do not code for functional proteins[5,15]. In Fig. 3 we compare the CM300 elution patterns of total human lens extract (bottom) with the γ-crystallin fraction (top). To identify the original gene products we used very young human lenses, because human

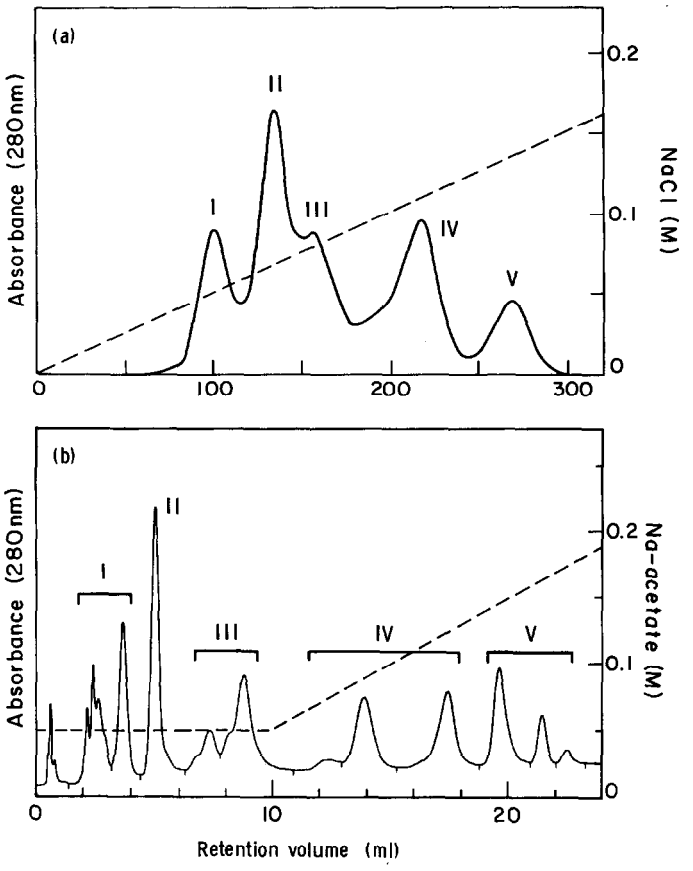

Fig. 2. Cation-exchange chromatography of dogfish total γ-crystallins. (a) SP-Sephadex; sodium acetate, pH 4.8, linear sodium chloride gradient (——); 10 mg protein applied: flow-rate, 12 ml/h; (b) SynChropak CM300; Tris–acetate, pH 6.0; 10% B isocratic for 10 min, 10 to 40% B linear gradient in 15 min; 50 μg protein applied; flow-rate, 1 ml/min. The SP-Sephadex fractions I–V were run individually on SynChropak CM 300 to determine the peak assignment indicated in b.

γ-crystallins are extremely susceptible to charge modification upon aging *in vivo*, generally becoming more acidic, presumably as a result of oxidation and mixed disulfide formation with glutathione and cysteine[2,9,10,19].

Four γ-crystallin peaks, γ_1–γ_4, are resolved at pH 5.5, yet they remain closely spaced suggesting only small differences in p*K* values of exposed His residues. This pH of optimal resolution is rather critical for human γ-crystallins. While pH 6.0 is optimal for γ-crystallins from most other species[14], some of the human γ-crystallins fail to bind at pH 6.0, thereby eluting with the α- and β-crystallins. The four γ-crystallin peaks in Fig. 3 (top) apparently do not represent the four different gene products; our amino acid analysis has shown that only two γ-crystallin genes are expressed significantly in young human lens[15]. These gene products presumably correspond to peaks γ_2 and γ_4 in Fig. 3, while the additional components in the elution profile are *in vivo* or *in vitro* modified forms.

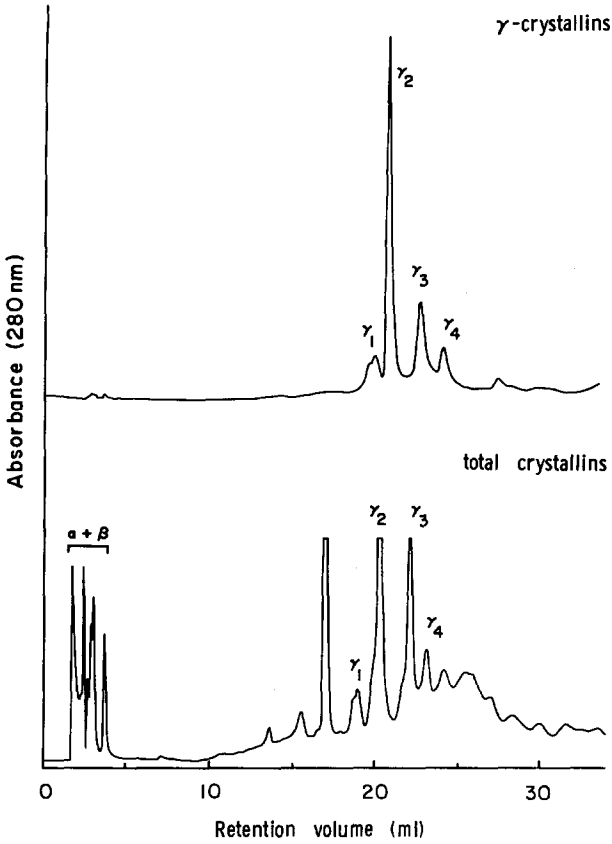

Fig. 3. SynChropak CM300 HPLC of human γ-crystallins (top) and human total lens extract (bottom). Elution conditions: Tris–acetate, pH 5.5; 0 to 30% B linear gradient in 30 min; flow-rate, 1 ml/min.

The same four γ-crystallin peaks can also be identified in the elution pattern of total lens extract (Fig. 3, bottom), despite a small shift in retention volume. However, the elution pattern of total extract is much more complex in the γ-crystallin region. It is not clear as yet whether the additional peaks between 10 and 30 ml represent α-, β- or γ-crystallins. We do know that β_s-crystallin, which is related to the γ-crystallins with respect to size, charge and amino acid sequence[10,15,20,21], is eluted as multiple peaks in the 10–30 ml region. In fact, the large peak at 17 ml may represent a β_s-crystallin since this protein is rather abundant in young human lens, in contrast to young rat lens[3,10,15,21].

SynChropak CM300 elution profiles of γ-crystallins from older human lenses (not shown) are even more complex, which is in agreement with electrophoretic studies[10,19]. Nevertheless, this HPLC procedure was extremely valuable in determining, for the first time, which γ-crystallin gene products are preferentially synthesized *in vivo*[15].

Bovine. In our initial communication[14] we reported the dependence on pH of the separation of bovine nuclear γ-crystallins on SynChropak CM300. A pH of

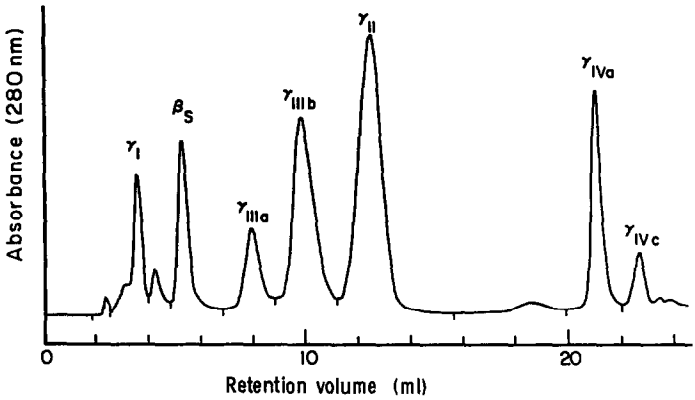

Fig. 4. SynChropak CM300 HPLC of bovine γ-crystallins. Monomeric crystallins from calf lens (14 days old) were applied. Elution conditions: Tris–acetate, pH 6.0; 10% B isocratic for 10 min, 10 to 40% B linear gradient in 15 min; flow-rate, 1 ml/min. Individual peaks were collected for amino acid analysis (Table I).

6.0–6.5 was recommended for optimal resolution. In Fig. 4 we now present a further improved separation of bovine γ-crystallins from total lens at pH 6.0. Seven well resolved, symmetrical peaks are seen and the position of bovine β_s-crystallin is now also clearly manifested.

TABLE I

AMINO ACID COMPOSITIONS OF BOVINE γ-CRYSTALLINS

The amino acid compositions are given in residues per mole of polypeptide. Numbers in parentheses give the integral number of residues obtained from amino acid sequences of γ_{II} (ref. 23) and β_s (ref. 20). Cys and Trp were not determined experimentally, but the compositions presented are based on a constant four Trp residues per polypeptide, seven Cys for γ_{II} (refs. 23 and 24), six Cys for β_s (ref. 20) and five Cys for γ_{III}- and γ_{IV}-crystallins[24].

Amino acid	*Residues per mol of*					
	γ_{II}	γ_{IIIa}	γ_{IIIb}	γ_{IVa}	γ_{IVc}	β_s
Asx	19.1 (19)	17.7	18.6	19.1	20.5	16.4 (16)
Thr	4.6 (5)	3.2	2.1	4.9	3.1	5.7 (5)
Ser	12.8 (13)	14.9	13.2	13.8	14.2	12.4 (12)
Glx	19.4 (19)	21.4	20.5	19.1	16.5	23.4 (22)
Pro	7.5 (8)	6.6	6.4	6.0	5.6	8.1 (7)
Gly	14.5 (14)	13.7	15.1	14.5	14.7	14.8 (14)
Ala	2.6 (2)	4.0	3.9	3.5	4.8	8.5 (9)
Val	6.1 (6)	6.9	7.0	6.5	5.4	9.1 (8)
Met	6.4 (7)	3.7	3.6	3.6	3.6	4.8 (6)
Ile	5.8 (6)	7.5	7.6	6.6	6.5	6.3 (7)
Leu	13.3 (13)	11.4	14.8	12.1	15.8	11.0 (10)
Tyr	14.5 (15)	13.4	15.2	14.5	16.2	10.2 (12)
Phe	9.1 (9)	9.0	6.1	9.2	5.9	9.9 (10)
His	5.2 (5)	7.9	7.2	8.1	7.0	6.4 (7)
Lys	2.0 (2)	1.9	2.3	1.2	1.0	7.2 (9)
Arg	20.1 (20)	21.0	20.5	21.4	23.0	12.9 (13)

The separated components were collected individually, and Table I presents their amino acid compositions, with the exception of γ_I. Unambiguous identification of gene products is only possible for two components, because the complete amino acid sequences are only known of bovine γ_{II} (refs. 22 and 23) and β_s-crystallin[20], both deduced from nucleotide sequences. In Table I the deduced amino acid compositions (in parentheses) of γ_{II} and β_s are compared with the measured compositions, and it is evident that we have correctly identified both components in the Fig. 4 elution profile. An interesting spin-off of these results is that our amino acid composition of γ_{II} supports the notion[23] that position 119 is Thr rather than Ser (ref. 22).

Our amino acid compositions of γ_{IIIa}, γ_{IIIb} and γ_{IVa} are in excellent agreement with other literature values for the two γ_{III}-crystallins[11] and the major γ_{IV}-crystallin[11,24]. The compositions of components γ_{IIIa}, γ_{IIIb}, γ_{IVa} and γ_{IVc} are sufficiently different (in particular their Thr, Glx, Leu, Tyr, Phe and Lys contents) to tentatively conclude that these peaks each represent a different gene product. For a more definitive identification we must await further isolation and characterization of the

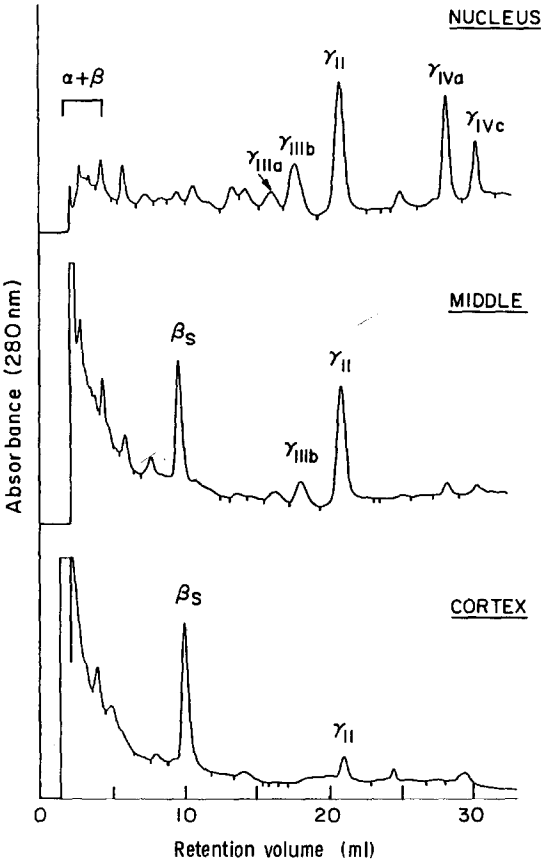

Fig. 5. SynChropak CM300 HPLC of total lens extracts from microsections (cortex, middle, nucleus) of bovine lens (5-years old). Elution conditions: Tris–acetate, pH 6.0; 5 to 10% B linear gradient in 2.5 min, 10% B isocratic for 10 min, 10 to 40% B linear gradient in 15 min; flow-rate, 1 ml/min.

other bovine γ-crystallin genes. A recently reported partial amino acid sequence, lacking the 17 C-terminal residues[23], appears to correspond to our γ_{IIIb} component, based again on comparison of amino acid compositions.

Two applications of the SynChropak CM300 separation of bovine γ-crystallins will now be presented.

Applications

Differential synthesis and aging. We have analyzed the distribution of γ-crystallins in lens microsections from a five-years old cow, as shown in Fig. 5. In the analysis of total lens extract we add a 2.5-min gradient elution (5 to 10% B) at the start to reduce interference with the breakthrough peak containing α- and β-crystallins[14].

Only three of nine concentric lens layers are shown, namely the innermost (nucleus), the fifth layer (middle) and the outermost layer (cortex). The γ-crystallin distributions in these layers reflect various stages of differential synthesis during lens development[3,7], since protein synthesis occurs only in the outer lens layers and very little protein turnover occurs during the concentric growth of the eye lens. From Fig. 5 we note a major shift in synthesis from γ-crystallins (inner nucleus) to β_s-crystallin (outer cortex) during lens maturation. We conclude that β_s-crystallin represents less than 5% of monomeric crystallins synthesized in early prenatal development (Fig. 5 and ref. 14), but more than 90% at the age of 5 years postnatal. At an intermediate age (middle layer) γ_{II}- and β_s-crystallin are the two predominant species synthesized, while synthesis of γ_{III}- and particularly γ_{IV}-crystalllins is greatly reduced.

In addition, information can be obtained on post-synthetic charge modification of γ-crystallins by comparing the inner layers with corresponding layers from young lenses, which we reported previously[14]. The top profile in Fig. 5 representing the oldest γ-crystallins has a large number of additional, more acidic components compared to young lens nucleus (Fig. 3 of ref. 14). While these are undoubtedly age-modified forms of the five main γ-crystallin species, the residual portions of these five components closely resemble the original distribution in young lens nucleus, suggesting that no selective charge modification occurs upon *in vivo* aging of bovine γ-crystallins. Finally, we note that the near absence of soluble α- and β-crystallins in the old nucleus (Fig. 5, top) compared to young nucleus[14] is indicative of selective insolubilization of α- and β-crystallins with aging.

Chemical modification. A second application tested is the *in vitro* oxidation by hydrogen peroxide of γ-crystallins in young bovine lens, in an experiment which simulates oxidative insult to lens proteins during cataract formation[1,2,9,10]. Subsequent analysis on SynChropak CM300 of total lens extracts, as shown in Fig. 6, shows the extent of charge modification of each individual $\gamma(+\beta_s)$-crystallin as a function of hydrogen peroxide concentration. The original γ-crystallin peaks disappear progressively with a concomitant increase of more acidic, earlier eluting components. Table II shows that β_s- and γ_{II}-crystallin are more susceptible to oxidative modification than the γ_{III}- and γ_{IVa}-crystallins, which is in agreement with their higher Cys contents (Table I)[9,20,24,25]. The amount of protein which is too acidic to bind to this column increases from 74.2 to 92.5% upon oxidation. Further details of this experiment, including analysis of charge modification by isoelectric focusing, is presented elsewhere[18].

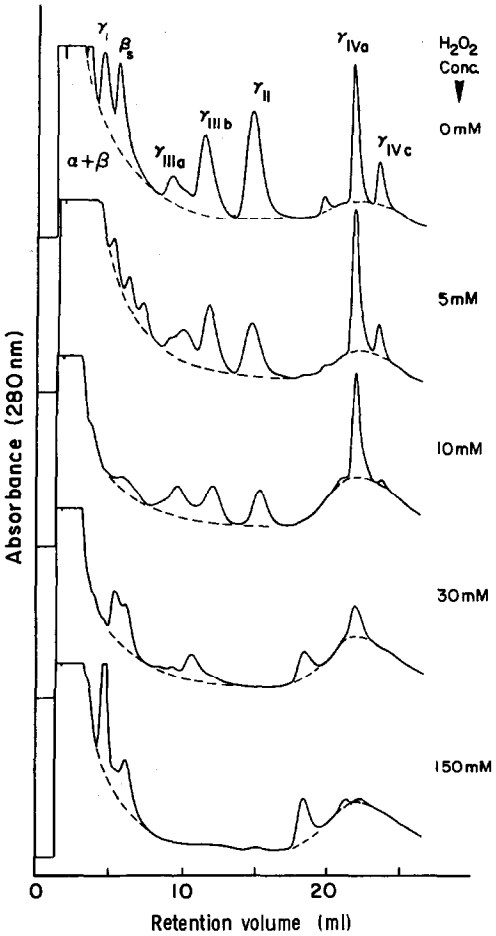

Fig. 6. SynChropak CM300 HPLC analysis of hydrogen peroxide induced modification of γ-crystallins in bovine lens. Total lens extract was applied. Elution conditions as in Fig. 4. The broad peak between 20–25 ml is a baseline artifact of the salt gradient.

CONCLUSIONS

Cation-exchange HPLC analysis of γ-crystallins on SynChropak CM300 offers greater speed, resolution and discrimination of peak heterogeneity than the previously employed low-pressure cation-exchange chromatography methods. Using this HPLC procedure one can rapidly analyze variations in γ-crystallin distributions which arise through differential synthesis, aging and cataractogenesis. Moreover, individual gene products can be readily identified if their nucleotide or amino acid sequence are known. Upscaling should be feasible using a preparative SynChropak CM300 column. This approach should provide adequate amounts of individual, pure γ-crystallins for detailed chemical, physical and crystallographical characterization.

TABLE II

HYDROGEN PEROXIDE INDUCED CHARGE MODIFICATION OF BOVINE γ-CRYSTALLINS *IN VITRO*

Whole calf lenses were incubated for 24 h in solutions of varying hydrogen peroxide concentration. Lens extracts were analyzed on SynChropak CM300 as shown in Fig. 6, from which peak areas [expressed as absorbance (%) at 280 nm] were obtained by integration. No correction is made for differences in specific absorbance at 280 nm. The breakthrough peak corresponds to lens proteins which are not bound to the ion-exchange column, notably α- and β-crystallins. "Other" components are the γ_1-crystallin peak and all other unidentified peaks, including charge-modified components.

Hydrogen peroxide concentration (mM)	*Peak area (%)*							
	Break-through	β_s	γ_{IIIa}	γ_{IIIb}	γ_{II}	γ_{IVa}	γ_{IVc}	*Other*
0	74.2	2.5	1.5	4.7	8.0	4.4	1.7	3.0
5	83.1	0.5	1.9	3.3	4.3	4.9	0.9	1.1
10	85.4	0	2.7	2.9	3.2	4.6	0.2	1.0
30	91.2	0	1.8	0.3	0	2.1	0	4.6
150	92.5	0	0	0	0	0.1	0	7.4

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